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Molecular Characterization of Malaysian Marine Fish Species using Partial Sequence of Mitochondrial DNA 12S and 16S rRNA Markers

(Pencirian Molekul Spesies Ikan Laut Malaysia menggunakan Urutan Separa Penanda Mitokondria DNA 12S dan 16S rRNA)

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ABSTRACT

This study was conducted to characterize the selected marine fish species using partial sequence of mtDNA 12S and 16S rRNA gene. PCR amplification of 12S and 16S rRNA generated PCR amplicons at 350 and 440 bp lengths, respectively. Sequence analysis was performed using BioEdit software. Phylogenetic tree was constructed using MEGA software. Two reference species have been used namely Gobio gobio *(AB239596 and EF112528) and* Pentapodus caninus *(DQ533268 and DQ532933). Based on the result obtained, mtDNA 12S and 16S rRNA were found to be useful as molecular markers for fish species identification. These markers will provide correct identification of fish species when considered along with morphological characteristics.*

Keywords: Mitochondrial DNA; species identification; 12S rRNA; 16S rRNA

ABSTRAK

Kajian ini dijalankan untuk mencirikan spesies ikan laut terpilih dengan menggunakan urutan separa gen mtDNA 12S dan 16S rRNA. PCR amplifikasi 12S rRNA dan 16S rRNA masing-masing menghasilkan amplikon PCR pada 350 dan 440 bp panjang. Analisis urutan dilakukan dengan menggunakan perisian Bioedit. Pokok filogeni dihasilkan dengan menggunakan perisian MEGA. Dua spesies rujukan digunakan iaitu Gobio gobio *(AB239596 dan EF112528) dan* Pentapodus caninus *(DQ533268 dan DQ532933). Berdasarkan keputusan yang diperoleh, mtDNA 12S dan 16S rRNA didapati berguna sebagai penanda molekul bagi pengenalpastian spesies ikan. Penanda ini akan menyediakan pengenalan yang betul bagi spesies ikan apabila digunapakai bersama-sama dengan ciri morfologi.*

Kata kunci: Mitokondria DNA; pengenalpastian spesies; 12S rRNA; 16S rRNA

INTRODUCTION

DNA based markers are widely applied to offer a better resolution of systematic relationships among species, especially mitochondrial DNA (mtDNA) markers. According to Stoeckle (2003), mtDNA genes are becoming an attractive target since these genes are shared across various taxa (species) and do not contain introns that complicate amplification.

Animal mtDNA genome is a single, double-stranded, circular, multiple copies and comprises of 37 genes, consisting of 13 protein-coding genes, two ribosomal RNAs (12S and 16S rRNA) and 22 transfer RNAs (tRNAs) (Meyer 1993). Its size varies from 16 to 19 kilo base pair in different species of animals. Mitochondrial DNA has been proved to be a powerful tool in fish species identification (Lemer et al. 2007; Rubinoff et al. 2006; Teletchea 2009), fish stock maintenance (Greig et al. 2005) and seafood control (Quinteiro et al. 1998). Mitochondrial 12S and 16S rRNA exhibit variation between species and within the species the sequence is stable.

Mitochondrial DNA 12S and 16S rRNA genes cover 1/16 and 1/10 of entire mitochondrial genome, respectively (Yang et al. 2014). Due to high mutation rate of mtDNA in most animals, it contributes to a significant amount of sequence variation in identification of closely related species (Yang et al. 2014).

DNA based identification provides a better method for taxonomic identification of fish species by showing a divergence of different taxa (Mohd-Shamsudin et al. 2011). Di Finizio et al. (2006) stated that the specific morphological characters of fish are sufficient in identification of fish species, but problem occurred if processed food is involved. Therefore the application of mtDNA as molecular genetic markers has been successfully applied to distinguish different species since this method is less expensive and reliable.

Mitochondrial DNA 16S rRNA gene analysis offers the most direct approach (direct sequencing) in differentiating marine animals, plant and bacteria (Baharum & Nurdalila 2012). It has been reported that mtDNA 12S and 16S rRNA genes have been used extensively as molecular markers to categorize mammals, birds, shrimp and other species (Kitano et al. 2007; Yang et al. 2014). DNA sequence of 12S and 16S rRNA showed high variability when compared

to other DNA markers since this region is reported as highly conserved (Wang et al. 2000). Greig et al. (2005) has utilized mtDNA 12S and 16S rRNA markers in North Atlantic Ocean sharks and found that within the species the sequence variability is very less compared to between species and it was suggested that these two markers are suitable for species identification.

Due to the features of mitochondrial ribosomal gene, the application of this gene as a genetic marker has become important due to highly conserved between closely related species. Therefore the genetic relationship of the selected marine fish species were studied and characterized using partial sequence of mtDNA 12S and 16S rRNA genes.

Materials and Methods

Sample

The marine fish used in this study were *Atule mate, Alepes vari, Alepes kleinii, Alepes djedaba, Parastromateus niger, Pristipomoides multidens, Pampus argenteus, Trachinotus blochii, Nemipterus bipunctatus* and *Nemiptereus furcosus*. These fish species were obtained from Pasar Siti Khadijah Market, Kota Bharu. The scientific name for selected fish species was obtained from the book entitled 'Marine Fishes and Fisheries of Malaysia and Neighboring Countries' (Mohsin & Mohd 1996) and also confirmation with reference to authenticated of species was done by consulting the Fisheries Department, Kota Bharu.

Genomic DNA Extraction

The parts of tissue or gills disserted and the fish species were preserved in absolute ethanol for longer storage. Prior to DNA extraction, fish samples must be washed with phosphate buffered saline (PBS) for three times to clean from ethanol. The genomic DNA extraction was performed using QIAamp tissue kit (Qiagen, Valencia, CA) and followed protocols as recommended by manufacturer. The integrity of the extracted DNA was inspected by agarose gel electrophoresis. The extracted DNA was quantified using spectrophotometer prior storage at -20°C.

PCR Amplification of 12S rRNA, 16S rRNA and Cytochrome b Gene

In the present study, published universal primers were used to amplify partial sequence of 12S rRNA (L1067F: 5'-AAA CTG GGA TTA GAT ACC CCA CTAT-3' and H1478R: 5'-GAG GGT GAC GGG CGG GCG GTG TGT-3' and 16S rRNA (L2510F: 5'-CGC CTG TTT ATC AAA AAC AT-3' and H3080R: 5'-CCG GTC TGA ACT CAG ATC ACG T-3') genes (Palumbi 1996). These primers generate PCR products of 350 and 550 bp for 12S and 16S rRNA, respectively.

The PCR mixture was amplified in 20 uL consisting of 1 uL of 10 pmol of each primer (Sigma, USA), 0.32 uL of 10 mM dNTPs (Bioline, USA Inc), 1X PCR buffer (Bioline, USA Inc), 2 uL of 25 mM MgCI2 (Bioline, USA Inc) and 1 U of Taq DNA Polymerase (Bioline, USA Inc). The reaction conditions are 95°C for 3 min followed by 30 cycles of 95°C for 30 s; 61°C (12S rRNA)/58°C (16S rRNA) for 1 min; 72°C for 1 min and a final extension at 72°C for 5 min. All PCR amplicons were checked by electrophoresis on 1% agarose gel and then was purified using PCR purification kit (Qiagen, USA). The purified PCR product was adjusted to 20-30 ng/uL of concentration using sterile deionized distilled water for further used in sequencing.

Direct Sequencing

A total of 10 µL sequencing reaction was prepared consisting of purified PCR product, 3.3 pmol of primers and 1:8 ABI BigDye® Terminator versions 3.1. Amplification was performed on GeneAmp PCR System 9700 (Applied Biosystem). The following thermal cycle condition was used at 96°C for 1 min, followed by 25 cycles of 96°C for 10 s, 50°C for 5 s, 60°C for 4 min and final hold at 4°C. The sequencing reaction was purified by ethanol precipitation prior to sequencing on ABI the 3130*xl* Genetic Analyzer.

Analysis of the Sequences

The polymorphisms reported in this study were analyzed using MEGA 4 (www.megasoftware.net) and BioEdit ver. v7.2.5 software (www.mbio.ncsu.edu) against the reference sequences obtained from GeneBank (www.ncbi.nlm.nih.gov/pubmed). The presence of polymorphisms such as transition, transversion, insertion and deletion were recorded. The neighbor-joining tree was constructed based on Kimura 2-parameter model using MEGA 4 software.

Results and Discussion

In this study, we have adopted the universal primers introduced by Palumbi (1996) to address the mitochondrial ribosomal gene 12S and 16S rRNA. It has been proved that the regions residing in the 12S and 16S rRNA loci in the mitochondrial genome among mammals are more strictly conserved than the region in the cytochrome b locus (Kitano et al. 2007; Palumbi 1996). These regions are located in both loop and stem portions in the secondary structure. The double-strand stem portion occupies 57 and 72% of the conserved regions of the 12S and 16S rRNA loci, respectively (Burk et al. 2002; Springer & Douzery 1996). It is likely that these conserved regions are functionally important. Using the highly conserved regions in this study, we were able to amplify DNA fragments 350 and 550 bp for 12S and 16S rRNA, respectively.

Base composition analysis of mtDNA 12S and 16S rRNA of fish species showed that the GC content was found to be higher in the mtDNA 12S than 16S rRNA. Higher sequence similarities with value more than 79% was observed between fish species *Nemipterus furcosus* and reference *Pentapodus caninus* for mtDNA 16S rRNA.

Higher number of polymorphisms were observed in mtDNA 16S than 12S rRNA as expected since the

amplicon lengths for 16S rRNA is longer than 12S rRNA. Higher percentage of insertion- deletion (32.98%) was observed among fish species with reference to *Pentapodus caninus* of mtDNA 16S rRNA. Sequence analysis indicated that *Atule mate* (234) and *Alepes djedaba* (233) showed the highest polymorphisms for 16S rRNA. The lowest polymorphisms were observed in *Alepes kleinii* (70) and *Alepes djedaba* (71) for 12S rRNA and *Nemipterus bipunctatus* 16S rRNA. More polymorphism was observed in *Nemipterus furcosus* compared with other fish species for 12S rRNA (Table 1). Lower numbers of polymorphism in *Pristipomoides multidens* for 16S rRNA suggest that this fish species share high percentage of similarity with the reference fish sample.

Genetic relationship among the fish species was examined using phylogenetic tree. A rooted neighbor joining tree using Kimura 2-parameter was constructed with bootstrapping of 1000. Phylogenetic tree of mtDNA 12S rRNA showed two clusters that were branching from a reference *Pentapodus caninus* (DQ533268) (Figure 1). The first cluster with bootstrapping value of 58% consisting of *Alepes* sp, *Nemipterus furcosus, Parastromateus niger, Trachinotus blochii, Nemipterus bipunctatus, Atule mate* and *Pristipomoides multidens*. The other cluster consist only *Pampus argenteus*. Study also showed *Gobio gobio* which is an outgroup species (AB239596) was rooted closely to *Pristipomoides multidens* (Figure 1).

Similar pattern of phylogenetic tree was observed in mtDNA 16S rRNA as mtDNA 12S rRNA except the value of bootstrapping much better in phylogenetic tree constructing mtDNA 16S rRNA than 12S rRNA (Figure 2). This suggests that the ribosomal genes can be used as genetic markers to distinguish fish species. *Alepes kleinii* and *Alepes djedaba* was found to have closer genetic relationship such as both share the same branch in phylogenetic tree, with a bootstrap value of 97% (Figure 1) and 99% (Figure 2).

Nemipterus furcosus is rooted distantly from *Nemipterus bipunctatus* though they are from the same genus. This indicates that *Nemipterus furcosus* and *Nemipterus bipunctatus* are differing from each other in terms of genetic composition.

Monophyletic clade was seen in *Alepes* sp. because they are from the same genus. *Pampus argenteus* (Figure 1) and *Trachinotus blochii* (Figure 2) was basal to other fish species, although bootstrap values were not strong enough to support the relationships. *Atule mate* and *Nemipterus bipunctatus* was found closely related in both

TABLE 1. Total polymorphisms observed in eight fish species of mtDNA 12S and 16S rRNA genes using BioEdit software

Species	Total polymorphisms	
	12S rRNA	16S rRNA
Atule mate	106	234
Alepes vari	75	220
Alepes djedaba	71	233
Alepes kleinii	70	205
Parastromateus niger	84	191
Trachinotus blochii	82	214
Nemipterus bipunctatus	225	79
Nemipterus furcosus	251	112
Pampus argenteus	89	205
Pristipomoides multidens	171	43

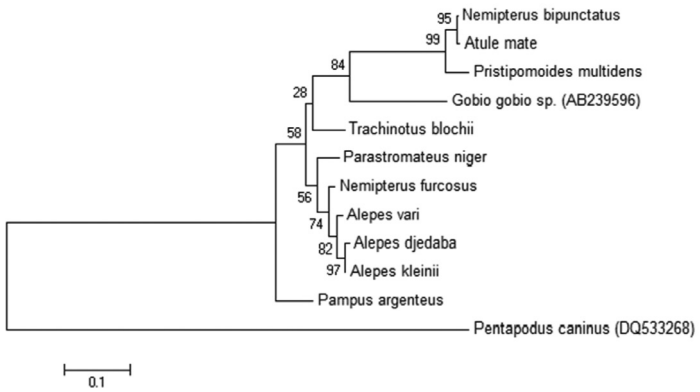

FIGURE 1. Neighbor joining tree of mtDNA 12S rRNA of fish species

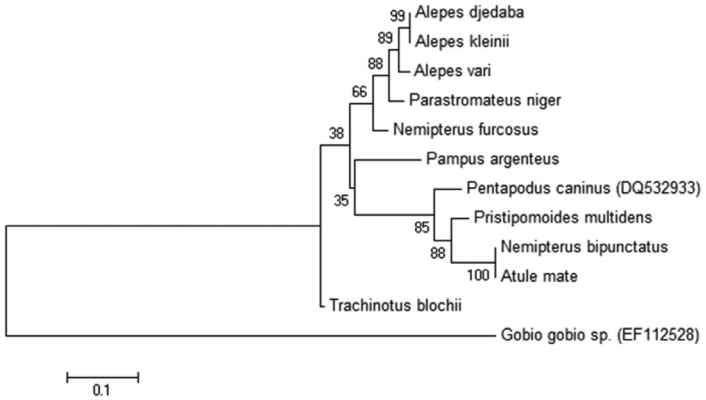

FIGURE 2. Neighbor joining tree of mtDNA16S rRNA of fish species

phylogenetic trees by sharing the same branch together with bootstrap value 99% (Figure 1) and 100% (Figure 2). Though they are from different genus they shared the same branch exhibiting closer genetic.

CONCLUSION

The present study successfully sequence partial mtDNA 12S rRNA and 16S rRNA from selected marine fish species. As stated by Kitano et al. (2007) direct sequencing of the PCR products allowed comparative analysis of the unknown DNA sequences and those already deposited in the databases by using the BLAST program. This study showed, though some fish species were from the same genus, the analysis of phylogenetic tree showed that they are genetically distant from each other. In addition, this study also showed that the application of molecular method especially mtDNA marker was successfully characterized and identified fish species. This suggest that mtDNA 12S rRNA and 16S rRNA also suitable as DNA DNA genetic markers by providing other alternative method in fish characterization since the DNA data generated is more reliable and convincing compared to morphological characterization.

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REFERENCES

Baharum, S.N. & Nurdalila, A.A. 2012. Application of 16S rDNA and cytochrome b ribosomal markers in studies of lineage and fish populations structure of aquatic species. *Mol. Biol. Rep*. 39: 5225-5232.

Burk, A., Douzery, E.J.P. & Springer, M.S. 2002. The secondary structure of mammalian mitochondrial 16S rRNA molecules: Refinements based on a comparative phylogenetic approach. *J. Mamm. Evol*. 9: 225-252.

Di Finizio, A., Guerriero, G., Russo, Gian, L. & Ciarcia, G. 2006. Identification of gadoid species (Pisces, Gadidae) by sequencing and PCR-RFLP analysis of mitochondrial 12S and 16S rRNA gene fragments. *Eur. Food Res. Technol*. 225(3): 337-344.

Greig, T.W., Moore, M.K., Woodley, C.M. & Quattro, J.M. 2005. Mitochondrial gene sequences useful for species identification of western North Atlantic Ocean sharks. *Fishery Bulletin* 103: 516-523.

Kitano, T., Umetsu, K., Tian, W. & Osawa, M. 2007. Two universal primer sets for species identification among vertebrates. *Int. J. Legal Med*. 121: 423-427.

Lemer, S., Aurelle, D., Vigliola, J.D. & Borsa, P. 2007. Cytochrome b barcoding, molecular systematics and geographic differentiation in rabbitfishes (Siganidae). *C. R. Biol*. 330(1): 86-94.

Meyer, A. 1993. Evolution of mitochondrial DNA in fishes. *Biochemistry and Molecular Biology of Fishes* 2: 1-38.

Mohd-Shamsudin, M.I., Fard, M.Z., Mather, P.B., Suleiman, Z., Hassan, R., Othmana, R.Y. & Bhassua, S. 2011. Molecular characterization of relatedness among colour variants of Asian Arowana (Scleropages formosus). *Gene* 490: 47-53.

Mohsin, A.K.M. & Mohd, A.A. 1996. *Marine Fishes & Fisheries of Malaysia and Neighbouring Countries*. Serdang: Universiti Pertanian Malaysia Press.

Palumbi, S.R. 1996. Nucleic acids II: The polymerase chain reaction. In *Molecular Systematics*. 2nd ed., edited by Hillis, D.M., Moritz, C. & Mable, B.K. Sunderland: Sinauer. pp. 205-247.

Quinteiro, J., Sotelo, C.G., Rehbein, H., Pryde, S.E., Medina, I., Perez-Martin, R.I., Rey-Mendez, M. & Mackie, I.M. 1998. The use of mtDNA direct PCR-Sequencing and PCR-RFLP methodologies in species identification of canned tuna. *Journal of Agricultural Food Chemistry* 46: 1662-1669.

Rubinoff, D., Cameron, S. & Will, K. 2006. A genomic perspective on the shortcomings of mitochondrial DNA for 'barcoding' identification. *J. Hered*. 97: 581-594.

Springer, M.S. & Douzery, E. 1996. Secondary structure and patterns of evolution among mammalian mitochondrial 12S rRNA molecules. *J. Mol. Evol*. 43: 357-373.

Stoeckle, M. 2003. Taxonomy, DNA, and the bar code of life. *BioScience* 53(9): 796-797.

Teletchea, F. 2009. Molecular identification methods of fish species: Reassessment and possible applications. *Rev. Fish Biol. Fish* 19: 265-293.

Wang, H-Y., Tsai, M-P., Tu, M-C. & Lee, S-C. 2000. Universal primers for amplification of the complete mitochondrial 12S rRNA gene in vertebrates. *Zoological Studies* 39(1): 61-66.
Yang, L., Tan, Z., Wang, D., Xue, L., Guan, M-X., Huang, T. & Li, R. 2014. *Species Identification through Mitochondrial rRNA Genetic Analysis*. Scientific Reports 4: 4089. Accessed June 17, 2014. DOI: 10.1038/srep04089.

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